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INSULIN DERIVATIVES AND THEIR APPLICATION

Application No.: 63-83,912
Application Date: April 5, 1988

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S P E C I F I C A T I O N

1. Title of the Invention:

INSULIN DERIVATIVES AND THEIR APPLICATION

2. Claims:

(1) Insulin having fatty acids bonded to amine groups of amine acids of insulin B_1 or B_{29} chains.

(2) Insulin having fatty acids bonded to amine groups of amine acids of insulin B_1 and B_{29} chains.

(3) A therapeutic composition having an effective component in the form of a pharmaceutically-allowable volume of a compound of Claim 1.

(4) A therapeutic composition having an effective component in the form of a pharmaceutically-allowable volume of a compound of Claim 2.

(5) A therapeutic composition of Claims 3 or 4 which is a diabetes-treating drug.

3. Detailed Description of the Invention

Field of the Invention

The present invention relates to insulin derivatives.

Description of Prior Art

Insulin is a polypeptide which originates in the isles of Langerhans situated in the pancreas and consists of 51 amino acid residual groups. Insulin is a hormone which controls the glucose level in the blood. If for any reason the secretion of insulin becomes below the normal, the level of glucose in the blood is increased and a disease known as diabetes is diagnosed. If a person suffering from diabetes ignores a high level of glucose in

his (her) blood, the condition of his (her) health may worsen and even end up in death. In order to correct the high level of glucose in the patient's blood, insulin must be administered to this patient. Insulin suitable for administering into a human body may be insulin extracted from the pancreas of a cow or a pig and converted into the human type by means of genetically rearranged bacillus coli, or by converting porcine insulin enzymically ..... (last line in the right column of page 1 of the Japanese original is illegible -- translator's ..... note).....

.....bovine insulin is comprised of alanine having B_{30} chain; and porcine insulin is formed of isoleucin and threonine having amino acids of A_8 and A_{10} chains. The human insulin, however, consists of theonine and isoluecin having amino acids of A_8 and A_{10} chains and threonine having amino acids of B_{30} chains.

The human, bovine, or porcine insulin is introduced into a patient's body by way of injection hypodermically or intramuscularly, and controls the blood sugar.

A person having diabetes should have insulin injections almost every day and at predetermined time intervals. Since injections are painful and are performed frequently, they cause local degeneration of the tissues and other problems.

In order to eliminate problems associated with introduction of insulin through injections, studies have been undertaken for developing new methods based on oral and rectal administration of insulin.

All new methods which resulted from these studies are based on preparation of insulin together with absorption accelerating agents and protein enzymolysis inhibitors. Methods of preparing enzymolysis inhibitors and examples of their application are described by Danforth [this name was transliterated phonetically

from Japanese and may have different spelling -- translator's note] et al. in Endocrinology, 65, 175, 1978]; a method based on the use of oily emulsions prepared with the help of emulsifiers is described by Shichiri et al. in Acta Diabet. Lab., 15, 175, 1978; and liposome-based method is disclosed by Yoshida in EPA 140,085. A method described by M. Saffran in Canadian J. Biochem., 57, 548, 1979 is based on coating the insulin particles with a polymer for its release without secretion of digestive enzymes in the large intestine.

Long-release insulin for oral administration is known as saccharified insulin, described in the following US patents: No. 4,478,830, No. 4,478,746, No. 4,483,792, No. 4,489,063, No. 4,489,064, and No. 4,536,572. Such insulins are obtained by causing precipitation of crystals in various saccharified insulins from conventional injection-type insulin-containing substances which are unsuitable for long storage.

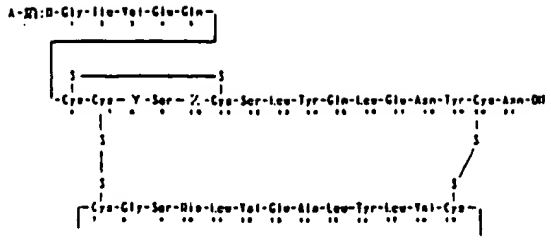
Problems Solved by the Present Invention

An objective of the present invention is to provide insulin derivatives suitable for preparation of pharmaceutically-allowable stable insulin drugs.

Means for the Solution of the Problems

The authors carried out a study which was paved the way to the development of a new liposoluble insulin which could be treated with fatty acids without a loss of the insulin activity and which showed a sugar-decreasing effect. Thus the authors arrived at the present invention.

New insulin derivatives of the present invention can be expressed by the following general formula:



(lower part could be missing - translator's note)

where R_1 and R_2 are identical or different fatty acid groups, X and Y are identical and are comprised of theonine or alanine, Z is either isoleucin, provided X and Y are threonines, or valine, provided X and Y are alanines; other symbols used in the above formula have the following meanings: Phe - phenyl alanine, Ile - isoleucine. Val - valine, Glu - glutamic acid, Cys - cystine, Ser - serine, Leu - leucine, Tyr - tyrosine, Asn - asparagine, His - histidine, Gly - glycine, Ala - alanine, Arg - arginine, Thr - threonine, and Pro - proline.

The compound of the invention is suitable for use as a drug for decreasing the level of blood sugar in diabetics.

The insulin of the invention works as a human, bovine, or porcine insulin.

It is advisable that the fatty acids which are bonded in the composition of the invention should have about 7 to 21 carbon atoms. The following is examples of such fatty acids: octanoic acid, peralgonic acid, capric acid, undecylic acid, lauric acid, tridecylic acid, myristic acid, pentadecylic acid, palmitic acid, heptadecylic acid, stearic acid, nonadecanoic acid, undecylenic acid, oleic acid, cetraric acid, elaidic acid, cetroleic [sic] acid, erucic acid, sorbic acid, linolic acid, linoleic acid, etc. The most preferable is palmitic acid.

A compound of the invention can be obtained, e.g., by the

following method:
(Step 1): synthesis of fatty acid activated ester;
(Step 2): treatment of insulin with p-methoxy benzoxy carbonylazide (pMZ) (pMZ insulin);
(Step 3): bonding pMZ-insulin to the fatty acid activated ester;
(Step 4): elimination of pMz groups;
(Step 5): isolation, purification, and storage.

The following is a more detailed explanation of each step.

Step 1, which is synthesis of activated esters is aimed at activating the carboxyl groups of fatty acids and increasing their reactivity. This is called for because fatty acids are not sufficiently reactive and would not be bonded as such to insulin. An example of a synthesized compound is N-hydroxy succinimide ester.

In step 2, the insulin is treated with p-methoxy benzoxy carbonylazide. As the amino acids contained in the A-chain insulin, especially amino groups of A_1 chain, are substituted by fatty acids, the insulin is subjected to the above-mentioned pMZ treatment for decreasing its activity and thus protecting the amino groups.

Step 3 is a reaction of bonding the activated fatty acid ester obtained in Step 1, to the pMZ-treated insulin obtained in Step 2. This procedure can be facilitated by being carried out with stirring at room temperature in a dimethylformamide-type solvent

In Step 4, pMZ which are protective groups introduced in Step 2, are eliminated by means of trifluoroacetic acid.

In Step 5, the product is subjected to gel filtration and high-speed liquid chromatography to obtain insulin of either B_1 or B_{29} chain type with fatty acids bonded to amino groups of amino

acids (i.e., the insulin with fatty acids bonded to R_1 or R_{29}), or else to obtain insulin having fatty acids bonded to amino groups of amino acids of both B_1 and B_{29} types (i.e., insulin with fatty acids bonded to R_1 and R_{29}).

The obtained insulin derivative is subjected to secondary freeze drying.... (the bottom line in the left lower column of page 3 of the Japanese original is missing -- translator's note)....

Reference Example 1. Preparation of Fatty Acid Activated Ester

Fifty (50) mM of palmitic acid and N-hydroxy succinimide (50 mM) were added to 150 mL of ethyl acetate, and while the mixture was cooled with ice-cold water, 50 mM of dicyclohexyl carbodiimide were added and stirred with the mixture for 24 hours. Upon completion of the reaction, the reaction solution was filtered, the solvent was evaporated, and the residual product was recrystallized with ethanol. The resulting product comprised a palmitic acid N-hydroxy succinimide ester.

Reference Example 2. Preparation of pMZ-Treated Insulin

1 mM of bovine insulin and 4 mM of p-methoxy benzoxy carbonylazide were dissolved in a mixed solvent composed of a 1N-solution of sodium hydrocarbonate, water, and dimethyl formamide (the components were mixed in a 2:3:4 ratio), and the solution was then stirred for 3 hours at room temperature. Upon completion of the reaction, the product was combined with 50% acetic acid, and the solvent was removed by distillation. The residual product was (recrystallized with ethanol and ....-..... (the bottom line in the right lower column of page 3 of the Japanese original is missing -- translator's note).....

1 mM of the pMZ-treated insulin was dissolved in dimethyl formamide. The solution was combined with 50 mM of palmitic acid N-hydroxy succinimide ester, and the components were then stirred for 3 hours at room temperature. Upon completion of the reaction, the solvent was removed by evaporation, the residual product was combined with anisole and trifluoroacetic acid, and the mixture was stirred for 1 hour under ice-cold conditions.

The trifluoroacetic acid was removed by evaporation, the residual product was combined with ether, and the precipitate formed in this process was separated by filtering. The residual product was then washed with ether.

In the next step, the product was dissolved in 1N acetic acid, and the insulin fraction was condensed by subjecting the product to gel filtration with the aid of a column filled with "Cephadix-G-25" (trademark - transliterated from Japanese -- translator's note).

After freeze-drying of the obtained insulin fraction, it was dissolved in a mixed solvent composed of acetonitrile and a 0.3% trifluoroacetic acid (the components were mixed in a 2:3 ratio). High-speed liquid chromatography showed that the product consisted of the following components: Lys-B_{29} palmitoyl insulin (pal-1), Phe-B_{11} palmitoyl insulin (pal-2), Phe-B_1-Lys-B_{29} dipalmitoyl insulin (pal-3).

Results of high-speed chromatography are shown in Table 1.

Table 1. Amino Acid Analysis

計算値	インスリン 未変性物	脱アミノ化物	脱アミノ化 mo-インスリン pal-1	pal-2	pal-3
3	2.93	3.03	3.47	3.12	3.05
1	0.94	0.96	0.97	1.00	1.00
3	2.54	2.71	3.05	2.80	2.71
7	7.32	7.5	8.25	7.39	7.49
1	1.15	1.23	1.00	1.09	1.09
4	4.05	3.36	3.28	3.26	3.26
3	3.00	3.00	3.00	3.00	3.00
3	2.38	2.4	-	0.94	1.73
5	3.3	3.7	4.87	4.19	4.04
1	0.31	0.28	0.62	0.53	0.55
6	5.42	5.56	6.47	5.81	5.67
4	3.91	1.65	-	2.77	2.90
3	2.58	2.21	2.94	2.83	2.21
1	0.95	0.09	0.05	0.69	0.79
2	1.96	1.93	2.09	1.94	2.01
1	1.1	1.39	1.92	1.89	1.51

⑥ 診断アミノ酸

1 - insulin; 2 - calculated values; 3 - non-modified substance; 3 - deaminized substance; 5 - deaminized pal-insulin; 6 - diagnostic amino acid

The positions where fatty acids were bonded to insulin derivatives obtained by the herein described method were identified by the following technique: after deamination of the derivatives, the product was subjected to acidic decomposition, all peptide bonds were broken, and after decomposition into 51 amino acids, the product was analyzed in an amino acid analyzer.

The values obtained as a result of this analysis are shown in Table 1. As can be seen from this table, the non-modified substances have free amino groups in three positions. This result could not be detected by the amino-acid analyzer because of the loss of amino groups during deaminization. In those cases, however, where fatty acids were bonded, deaminization was not feasible, and therefore the bonding positions could be identified by comparing the deaminized substance with a biological insulin. The comparison showed that in many cases the fatty acids were bonded in one position only.

Experimental Example (Blood Sugar Decrease Effect

After 24-hour fasting, male rats of the Vistar line were immobilized and anesthesized by injecting pentabarbital into the lower back side of the body, and then were administered a test drug dissolved or suspended in 1N hydrochloric acid. The test substance was injected into the thigh intramuscularly or intravenously. The dosage was 100 μg per animal. After administering the drug, a blood sample was taken from the jugular vein, and the level of blood glucose was measured.

The r sults are shown in Fig. 2.

As can be seen from the drawings, the introduction of insulin derivatives of the invention (Pal-1 and Pal-2) significantly decreases the level of glucose in the blood.

4. Brief Description of the Drawings

Fig. 1 is a graph illustrating the results of high-speed liquid chromatography. Fig. 2 is a graph which shows a decrease in the blood sugar level under the effect of the insulin derivatives of the invention. (This last line is missing in the original and was translated on the basis of the drawing and the previous text -- translator's note).

FIG. 1

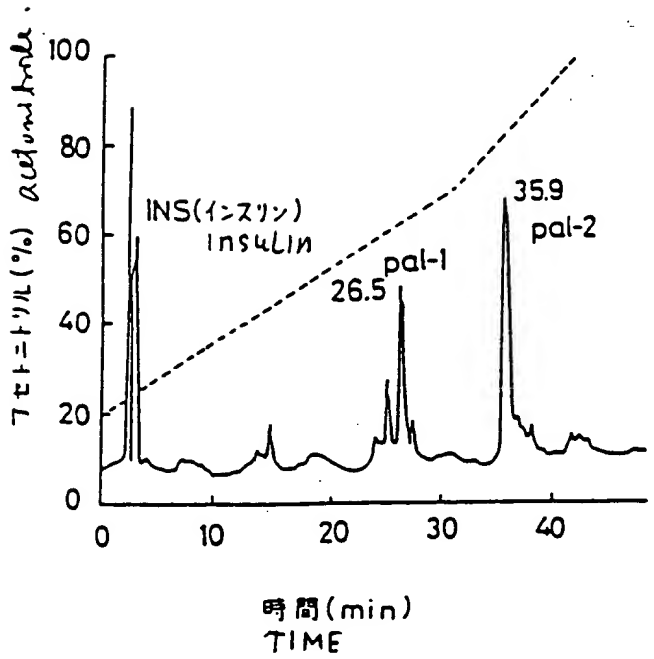


FIG. 2